0022-202X/83/8103-0204$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 81:204–208, 1983

Vol. 81, No. 3
Printed in U.S.A.

Identification of Chemoattractant Activity for Lymphocytes in Blister Fluid of Patients with Bullous Pemphigoid: Evidence for the Presence of a Lymphokine

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Bullous pemphigoid is characterized by the dermal infiltration of lymphocytes, which precedes the striking influx of eosinophils as the lesion evolves into the bullous phase. This finding prompted a search for chemoattractant activity for lymphocytes in the blister fluid of untreated individuals with bullous pemphigoid. We found such activity in the bullous fluids of 6 consecutive patients but not in a patient with pemphigus vulgaris. This lymphocyte chemoattractant activity separates into 4 peaks upon Sephadex G-100 chromatography and the peak of 56,000 daltons was further evaluated. Upon quaternary aminoethyl Sephadex-anion exchange chromatography this peak elutes at 4–8 ms and with preparative isoelectric focusing it demonstrates an isoelectric point of 8.6–9.0. This activity was susceptible to degradation by trypsin and neuraminidase, but was stable upon heating to 56°C for 30 min. Its chemoattractant activity is predominantly chemokinetic by checkerboard analysis. As defined by chromatography, stability, and functional characteristics, this activity is similar to a recently described human lymphocyte chemoattractant lymphokine. This finding suggests that products of activated lymphocytes are present in blister fluids of patients with bullous pemphigoid and may contribute to the early influx of lymphocytes in this disease.

Bullous pemphigoid is a disease characterized by blisters that form at the dermal-epidermal junction [1]. Affected individuals have a serum antibody directed against a basement membrane antigen [2–4] and complement deposition along the basement membrane [5–7]. The identification of activated complement proteins and complement-derived neutrophil chemotactic activity in blister fluids and of the deposition of the third component of complement (C3) along the basement membrane has led to the assumption that complement activation participates in the pathogenesis of the process [5]. This assumption is further supported by the characterization of functional, complement-fixing immune complexes in the diseased skin [8] and the in vitro demonstration of dermal-epidermal separation caused by the action of pemphigoid antibodies on normal human skin in the presence of complement and normal leukocytes [9]. The presence of presumptive mast cell-derived chemotactic activity for eosinophils in bullous fluid along with the demonstration of degranulated skin mast cells in the dermis has implicated the mast cell as a potential participating inflammatory cell either directly or, more likely, through the attraction of eosinophils, which are strikingly prominent as the bullous lesion develops [2,10–12]. However, rather than the eosinophil, the lymphocyte is the earliest infiltrating cell into the dermis [10]. This observation led us to a preliminary demonstration of lymphocyte chemoattractant activity (LCA) in bullous pemphigoid blister fluids [13].

In this study, a LCA in bullous pemphigoid blisters has been characterized as being physicochemically identical to a recently described human lymphocyte chemoattractant lymphokine [14,15]. It has a M_r of 56,000 by molecular sieve chromatography and is a cationic sialoprotein as evidenced by an isoelectric point of 8.6–9.0 and susceptibility to inactivation by trypsin and neuraminidase. The evidence that this product of activated lymphocytes is present in blister fluids of individuals with bullous pemphigoid may partially account for an early pathologic abnormality observed during assembly of the bullous pemphigoid lesion, namely the accumulation of lymphocytes.

MATERIALS AND METHODS

Medium 199 (M199) (Microbiological Associates, Bethesda, Maryland); bovine serum albumin (BSA) (Miles Laboratory, Inc., Elkhart, Indiana); Sartorius nitrocellulose 8-μm micropore filters (Sartorius Filters, Inc., Cherry Hill, New Jersey); polystyrene chemotactic chambers (Adaps Corp, Bedford, Massachusetts); ampholytes pH 3–10 (Bio-Rad Laboratories, Rockville Centre, New York); Ficoll-Paque, Sephadex G-100, and quaternary aminoethyl Sephadex (QAE Sephadex), (Pharmacia Fine Chemicals, Piscataway, New Jersey); Sprague-Dawley rats (Gofmoor Farms, Westboro, Massachusetts); nylon wool (Fenwal Laboratories, Division of Travenol Laboratories, Inc., Deerfield, Illinois); and neuraminidase and trypsin (Sigma Chemical Co., St. Louis, Missouri) were obtained from the manufacturers and suppliers.

Patient Population

The blister fluids of 6 patients with bullous pemphigoid, diagnosed by characteristic clinical appearance and by biopsies that showed typical changes on light microscopy and linear deposits of C3 at the epidermal-basement membrane zone as detected by immunofluorescence analysis, were studied. All patients were hospitalized and treated with saline compresses; some received antihistamines to control pruritus. None had received corticosteroids at the time that blister fluids were obtained. Blister fluids were aspirated from intact bullae in a sterile fashion into 10-ml syringes containing 1.0 ml acid citrate, pH 5.2, to serve as an anticoagulant. The cellular contents were removed by centrifugation and used in other studies. Blister fluids were stored

Manuscript received January 31, 1983; accepted for publication April 28, 1983.

Supported in part by a grant from the American Lung Association, and by grants HL-29544, HL-19717, AI-07722, AI-10356, AM-28388, and RR-05669 from the National Institutes of Health.

* Dr. Center was an Edward Livingston Trudeau Fellow of the American Lung Association and Recipient of NHLBI Young Investigator Award No. 24483.

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Abbreviations:
BSA: bovine serum albumin
C3: third component of complement
hpf: high-powered field(s)
LCA: lymphocyte chemoattractant activity(ies)
M199: Medium 199
M199-BSA: Medium 199 plus 0.4% bovine serum albumin
PBS: phosphate-buffered saline
QAE Sephadex: quaternary aminoethyl Sephadex

at 70°C in 1-ml portions until assessed for LCA. The histopathologic and immunopathologic descriptions of uninvolved skin, erythematous macules, plaques, and bullae of 5 of these patients have been described previously [10]. The blister fluid of a patient with typical pemphigus vulgaris was collected and stored in a similar manner. Blood from a normal volunteer was collected in acid citrate for use as a source of plasma.

Lymphocyte Migration

LCA was assessed by means of a modification [16] of a Boyden chamber [17] technique. Briefly, lymphocytes to be employed as the responding cell pool were obtained from the spleens of Sprague-Dawley rats. Spleens were teased apart with surgical forceps on plastic dishes to permit adherence of monocytes. The nonadherent cells were centrifuged on Ficoll-Paque cushions: and the mononuclear cells at the interface were aspirated, washed twice in M199, and suspended at a concentration of 4×10^6 cells/ml in M199 plus 0.4% BSA (M199-BSA). This cell suspension was routinely 95% lymphocytes as assessed by light microscopic evaluation of smears stained with Wright's, Giemsa's, or esterase stains.

To determine the LCA in any experimental sample, 4×10^6 lymphocytes in 1 ml M199-BSA were placed in the upper compartments of chemotactic chambers that utilized 8-μm nitrocellulose micropore filters to separate the cells from 1 ml of M199-BSA alone (control) or of M199-BSA containing experimental samples. To differentiate chemokinesis from chemotaxis, checkerboard analyses were performed in which experimental factors were introduced in varying concentrations into the lower compartment only, into both compartments, or into the upper compartment only of chemotactic chambers [18]. Migration experiments were carried out for 3 h at 37°C in a 5% CO_2 moist atmosphere. The filters were fixed, stained, dehydrated, and mounted according to standard histologic methods. Lymphocyte movement was quantitated by counting the total number of cells migrating beyond a distance of 50 μm at 10-μm intervals in 5 high-powered fields (hpf) in duplicate micropore filters. Results were calculated as mean cells/hpf ± S.E. Significance of data was determined by analysis of variance. For the convenience of comparison among experiments, data were expressed as a percentage of migration relative to control conditions; however, the baseline migration of cells/hpf is also noted for each experiment in the figure legends. All statistical analyses were performed on the raw data [19].

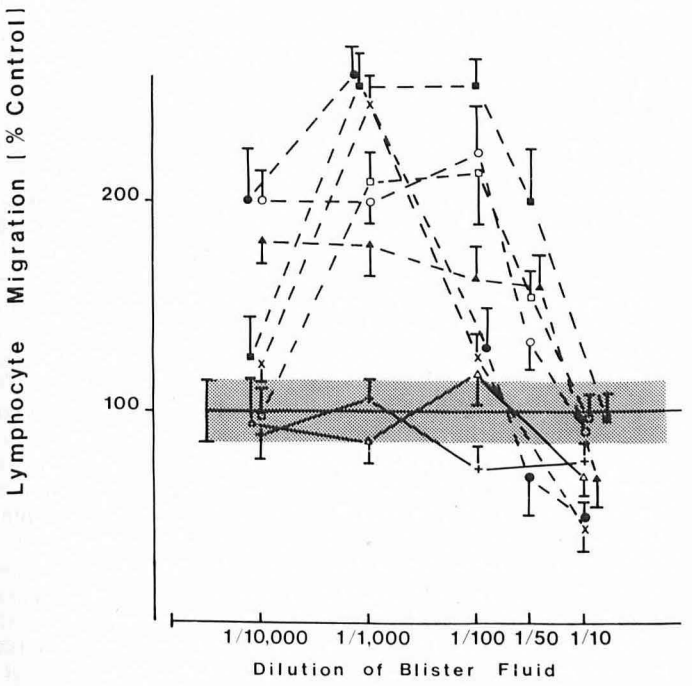

FIG 1. Lymphocyte migratory response to varying dilutions of the blister fluids of 6 patients with bullous pemphigoid (○- - -○, ●- - -●, □- - -□, ■- - -■, ×- - -×, ▲- - -▲), 1 patient with pemphigus vulgaris (+——+), and normal human plasma (△——△) placed in the lower compartment only of chemotactic chambers. Data are expressed as a mean percent of migration ± SE in response to control buffer alone (100%, *horizontal line*) which ranged from 8.9 to 12.1 cells/hpf. The fluids from each patient were assessed 3 different times. The *shaded area* represents the mean migration in response to control buffer ± 2 SE.

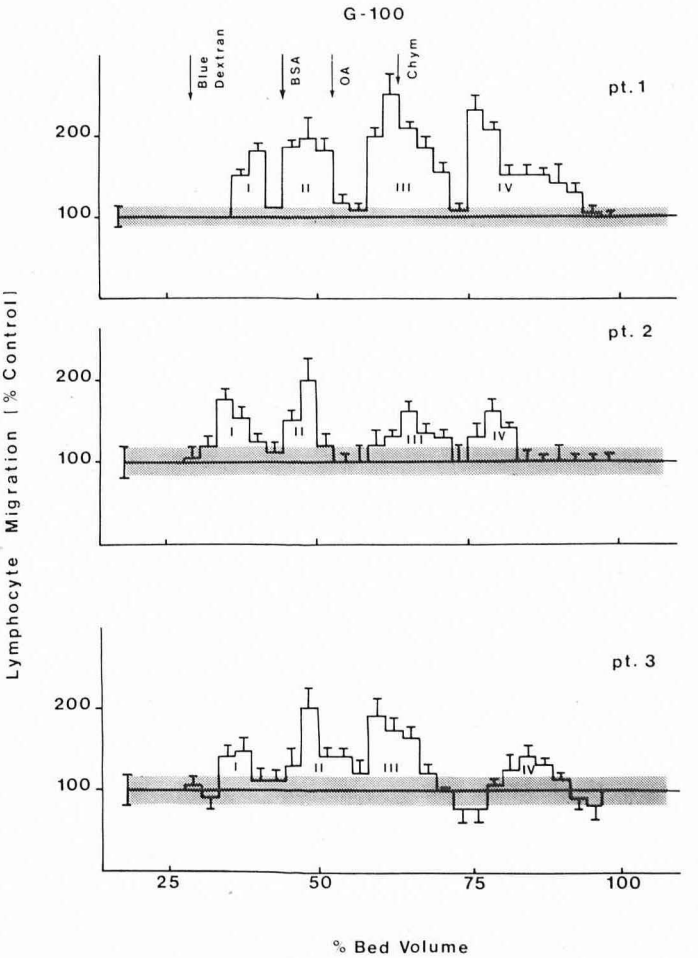

FIG 2. Lymphocyte migratory response to blister fluids of patients 1 (○- - -○), 2 (□- - -□) and 3 (×- - -×) from Fig 1 after Sephadex G-100 chromatography. Data are expressed as a mean percent of control migration ± SE (100%, *horizontal line*) which was 8.2 cells/hpf for the assay of patient 1; 9.1 cells/hpf for patient 2; and 10.2 cells/hpf for patient 3. The percents of bed volume are expressed on the *abscissa*. The *shaded area* represents the mean migration in response to control buffer ± 2 SE.

Chromatography

The chromatographic characterization of LCA from blister fluids was performed in an identical fashion to that previously described for the purification of lymphotactic lymphokines from human peripheral blood lymphocytes stimulated with concanavalin A [15]. Sephadex G-100 chromatography was performed at 4°C in a 1.5 x 90 cm column eluted with phosphate-buffered saline (PBS), pH 7.4, at 15 ml/min. Two-milliliter fractions were collected in plastic tubes. A 100-μl sample of every other fraction was placed in the lower chamber only of a chemotactic chamber, and the samples were assessed for chemoattractant activity for rat splenic lymphocytes. Fractions with chemoattractant activity were pooled and dialyzed against a 0.01 M phosphate buffer, pH 7.8. This pooled material was then applied to a 4-ml QAE Sephadex-anion exchange column equilibrated in the same buffer, washed with 2 bed volumes of buffer, and eluted with a linear 200-ml salt gradient to 1 M NaCl at 4°C. Four-milliliter fractions were collected and separately dialyzed against PBS, pH 7.4. Two hundred-microliter portions of each fraction were then evaluated for chemoattractant activity in duplicate. Fractions with chemoattractant activity were pooled, dialyzed against distilled water, lyophilized to effect a 10-fold concentration, and subjected to isoelectric focusing in sucrose with a Buchler apparatus (Fort Lee, New Jersey). Twenty-milliliter (10–40%

sucrose) gradients were generated with a Buchler gradient maker and a constant-flow pump containing ampholytes in the 3–10 pH range. One-milliliter fractions were eluted with a peristaltic pump, assessed for pH, and dialyzed against PBS, pH 7.4, for 24 h at 4°C to remove ampholytes and sucrose; a 250-μl sample of each fraction was placed in the lower compartment of duplicate chemotactic chambers and assessed for LCA.

RESULTS

Blister fluids from 6 consecutive patients were assessed for the presence of LCA by placing varying concentrations of anticoagulated fluids in the lower chamber of modified Boyden chambers (Fig 1). At a concentration of 1:100,000, no activity could be detected in any of the fluids. Increasing doses resulted in the expression of measurable LCA in each fluid over a wide concentration range. However, high concentrations (greater than 1:100) frequently resulted in an inability to measure the LCA. No LCA was present in plasma at similar dilutions; at concentrations of 1:100 or more a slight inhibitory effect was seen, similar to that noted with the blister fluids. Serial dilutions of blister fluid from a patient with typical pemphigus vulgaris (Fig 1) or from suction-induced blisters contained no activity. The fluid from a patient with contact dermatitis did contain significant amounts of LCA (data not shown).

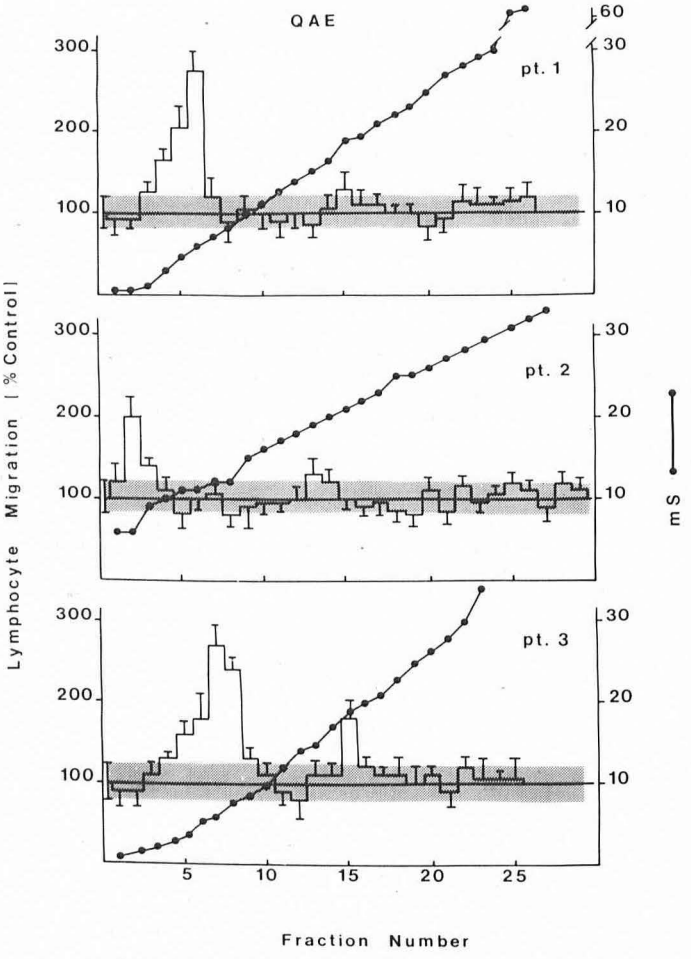

FIG 3. Sephadex QAE-anion exchange chromatography of lymphocyte chemoattractant activity in the Sephadex G-100 peak II from Fig 2 of patients 1, 2, and 3. Data are expressed as a mean percent of control migration ± SE (100%, *horizontal line*) which was 7.9 cells/hpf for the assay for patient 1, 13.2 cells/hpf for patient 2 and 10.1 cells/hpf for patient 3. The linear salt gradient (●——●) is expressed in mS; fraction numbers are shown on the *abscissa*. The *shaded area* represents the mean migration in response to control buffer ± 2 SE.

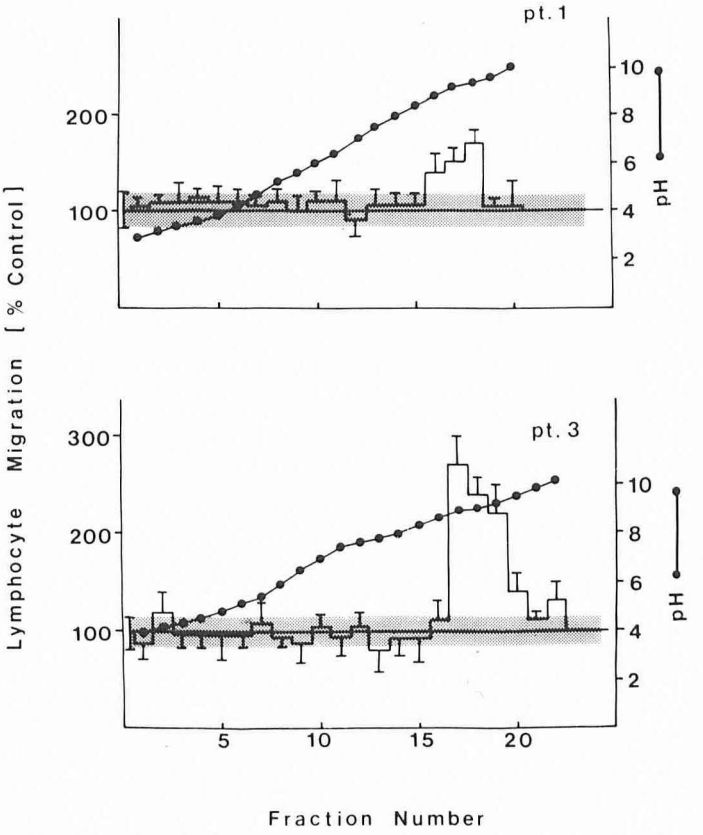

FIG 4. Isoelectric focusing of lymphocyte chemoattractant activity in the Sephadex QAE 4–8 ms peak from Fig 3 of patients 1 and 3. Data are expressed as a mean percent of control migration ± SE (100%, *horizontal line*) which was 13.4 cells/hpf for the assay for patient 1 and 10.6 cells/hpf for patient 3. The pH (●——●) of each fraction is depicted by the *diagonal line*. Fraction numbers are shown on the *abscissa*. The *shaded area* represents the mean migration in response to control buffer ± 2 SE.

In order to characterize the LCA further, 1.5-ml portions of bullous fluid from 3 of the 6 patients were separately subjected to Sephadex G-100 chromatography (Fig 2). This chromatography resulted in the identification of 4 distinct regions of LCA: the first filtered near the void volume; the second and third corresponded to M_rs of 56,000 and 25,000–30,000, respectively; and the last filtered near the bed volume. Plasma and pemphigus vulgaris blister fluid were also subjected to Sephadex G-100 chromatography to make certain that the absence of activity noted with serial dilutions was not due to the simultaneous presence of inhibitors. No LCA was uncovered by this maneuver. The region of LCA that eluted between the BSA and ovalbumin markers (peak II, 56,000 daltons) was further characterized for comparison with a lymphokine of similar size generated from human lymphocytes by antigens or mitogens [14,15].

Fig 3 depicts the QAE Sephadex-anion exchange chromatography of the second Sephadex G-100 peak of LCA from each of the 3 patients. The LCA eluted with application of the salt gradient, corresponding to an ionicity of 4–8 ms. The G-100 fraction from the bullous fluid of patient 3 had a second smaller peak of activity that eluted from QAE at 18 ms. The active fractions from the 4–8 ms region of each fluid were pooled, dialyzed, and subjected to isoelectric focusing in sucrose with ampholytes in the 3–10 pH range. The results obtained with fluids from patients 1 and 3 are displayed in Fig 4; assayable activity was identified in fractions corresponding to an isoelectric point at 8.6–9.0. Patient 2 had insufficient activity to be measured after focusing.

TABLE I. *Checkerboard analysis of lymphocyte chemoattractant activity (LCA) purified through the QAE Sephadex step*

Microliters of LCA below filter	Microliters of LCA above filter				
	0	25	50	100	200
0	100[a]	121 ± 13	171 ± 14[b]	210 ± 12[b]	228 ± 16[b]
25	98 ± 10	126 ± 10			
50	116 ± 14		181 ± 9[b]		
100	172 ± 15[b]			219 ± 12[b]	
200	205 ± 14[b]				232 ± 10[b]

[a] Values represent % of control migration ± SE, where control ranged from 7.5–8.8 cells/high-powered field in 3 experiments with LCA from patients 1, 2, and 3.

[b] Different from controls, $p < 0.05$.

TABLE II. *Chemical stability of lymphocyte chemoattractant activity (LCA) purified through the QAE Sephadex step*

Buffer control	100[a]
LCA (200 μl)	199 ± 10[b]
LCA + heat (56°C; 30 min)	201 ± 9[b]
LCA + neuraminidase (10^{-4} M)[c]	105 ± 11
LCA + trypsin (10^{-6} M)[d]	96 ± 8

[a] Values represent % of control migration ± SE where control ranged from 9.8–13.4 cells/high-powered field in 6 experiments—2 each from patients 1, 2, and 3.

[b] Different from control, $p < 0.05$.

[c] Neuraminidase (10^{-4} M) corresponds to 0.04 units/ml incubated with 200 μl LCA for 30 min at 37°C before chemotactic assay.

[d] Trypsin (10^{-6} M) corresponds to 2.1 units/ml incubated with 200 μl LCA for 30 min at 37°C; excess soybean trypsin inactivator was added at end of incubation before chemotactic assay.

The averaged results of the checkerboard analyses of the LCA from patients 1, 2, and 3 after Sephadex G-100 (peak II) and QAE Sephadex-anion exchange (4–8 ms) chromatography are presented in Table I. Lymphocyte migration is stimulated when portions of active QAE fractions are placed in either the cell compartment or the lower compartment. Since migration appears to be stimulated to a greater extent when the active fractions are incubated with the cells, the chemoattractant activity is predominantly chemokinetic. Further characterization (Table II) of similarly enriched chemoattractant activity (after Sephadex G-100 and QAE chromatography) from patients 1, 2, and 3 demonstrated it to be stable upon heating to 56°C for 30 min, but susceptible to biologic inactivation by trypsin (10^{-6} M) and neuraminidase (10^{-4} M).

DISCUSSION

A LCA that is present in the blister fluid of untreated patients with bullous pemphigoid (Fig 1) has been isolated and characterized as a 56,000-dalton cationic protein with an isoelectric point of 8.6–9.0 (Figs 2–4). This activity is predominantly chemokinetic in nature (Table I), is resistant to degradation by heat, and is sensitive to trypsin and neuraminidase treatment (Table II). By physicochemical and functional criteria, this activity is identical in each respect to a recently described human lymphotactic lymphokine [15,16] that is generated in culture by incubation of human peripheral blood lymphocytes with antigen, concanavalin A, or phytohemaglutinin.

The Sephadex G-100 molecular sieve chromatography reveals additional LCA present in the bullous fluid (Fig 2). Since LCA have been identified from mast cells [20], macrophages [21], and complement activation [22], all of which may be important in the pathologic process of bullous pemphigoid, these other LCA could be from any of these sources. None of these other LCA have been characterized sufficiently for comparison with the activities present in the bullous fluids. Any of these LCA might represent the initiating stimulus for the early lymphocytic infiltrate in bullous pemphigoid, or alternatively may represent a product of one of the inflammatory systems secondarily activated in this disease. The presence of a lymphokine, for example, may be a result of lymphocyte activation by products of tissue mast cells similar to those that are of basophil origin [23] or that arise from activated complement components [24].

The recognition of a putative lymphokine in blister fluids of patients with bullous pemphigoid suggests that other products of activated lymphocytes may also be present. In fact, a colony stimulating factor-like activity for eosinophils has recently been reported to be present in bullous pemphigoid blister fluids [25]. The finding of lymphokines in blister fluids, the observation in one study that the mononuclear cells of 6 of 7 patients with bullous pemphigoid produced macrophage migration inhibitory factor in response to autologous epidermal extracts [26], and the demonstration that lymphocytes are the earliest infiltrating cells in a prebullous site [10,13] suggest a role for lymphocyte-mediated hypersensitivity in the pathogenesis of bullous pemphigoid.

The authors wish to thank Laurie Beardsley for typing the manuscript.

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0022-202X/83/8102-0208$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 81:208–211, 1983

Vol. 81, No. 3
Printed in U.S.A.

Epidermal Cell-Induced Generation of Cytotoxic T-Lymphocyte Responses Against Alloantigens or TNP-Modified Syngeneic Cells: Requirement for Ia-Positive Langerhans Cells*

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The role of epidermal cells (EC) in the activation of T-cell proliferation is well established. In this study we asked whether EC can provide a stimulus resulting in the generation of genetically restricted T-cell cytotoxicity. For this purpose, C57Bl/6 or C3H/He highly purified, accessory cell-depleted responder splenic T lymphocytes, were stimulated in 5-day cell-mediated cytotoxicity cultures with mitomycin C-treated allogeneic or trinitrophenyl (TNP)-modified syngeneic EC, or, for control purposes, with unfractionated spleen cells (SC). Untreated and complement (C′)-treated EC induced strong cytotoxic T lymphocyte (CTL) activity in highly purified allogeneic T cells and, in analogy, TNP-modified EC led to the generation of TNP-self CTL, as tested in 4-h ^{51}Cr release assays against allogeneic or TNP-modified syngeneic EC or SC targets. These cytotoxic responses were comparable in magnitude to those seen with allogeneic or TNP-modified syngeneic SC stimulators. In contrast, alloreactive or TNP-self CTL responses were not generated when stimulating EC were depleted of Langerhans cells by pretreatment with anti-Ia monoclonal antibodies plus C′ or, for control purposes, when highly purified T-cell stimulators were used.

These results demonstrate that EC induce the generation of alloreactive and TNP-self CTL in the absence of Ia-positive splenic accessory cells and that Ia-bearing Langerhans cell are required for this process to occur.

Manuscript received November 2, 1982; accepted for publication March 29, 1983.

* Presented in part at the 43rd Annual Meeting of The Society for Investigative Dermatology, Inc., Washington, D. C., May 6–8, 1982.

This work was supported by Fonds zur Förderung der wissenschaftlichen Forschung project No. 3791, Vienna, Austria.

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Abbreviations:
C′: complement
CML: cell-mediated lympholysis
CTL: cytotoxic C lymphocyte(s)
EC: epidermal cell(s)
FCS: fetal calf serum
FITC: fluorescein isothiocyanate
LC: Langerhans cell(s)
NMS: normal mouse serum
SC: spleen cell(s)
TNBS: 2,4,6-trinitrobenzenesulfonic acid
TNP: trinitrophenyl

In the recent past it has been clearly shown that epidermal cells (EC), consisting of keratinocytes, melanocytes, and Langerhans cells (LC), can initiate cutaneous immune reactions. This is particularly true for LC. These dendritic cells comprise a small population (3–8%) of all EC, originate from a bone marrow-derived precursor, bear Fc-IgG and C3b receptor sites, synthesize Ia antigens, and are critically needed for EC-induced antigen-specific, allogeneic and mitogenic (L. A. Stingl, unpublished observation) T-cell proliferative responses (reviewed in [1]). Furthermore, murine EC induce the generation of cytotoxic T lymphocytes (CTL). This has been reported for allo-